Web extra appendices

Chiu et al, Non-invasive prenatal assessment of trisomy 21 by multiplexed maternal plasma DNA sequencing: large scale validity study

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Appendix 1.

Steps of quality control assessments adopted for maternal plasma DNA sequencing.

QC step No.	Analysis phase	Check item	Criteria for failure	Cases failed	Consequence
1	Before DNA extraction	Plasma sample quality	Collected after invasive obstetric procedure, processed 6 hours or more after venepuncture, less than 2 ml, haemolysed, ambiguous tube labels	46	Do not proceed to laboratory analysis.
2	After DNA extraction	Real-time PCR for the β-globin gene	No amplification	2	Do not proceed to DNA library preparation.
3	After sequencing library preparation	DNA detection by fluorometry, real-time PCR or electrophoresis in a bioanalyzer	No signal or no amplification	8	Do not proceed to sequencing.
4	After sequencing	Total number of unique perfectly matched sequenced reads	< 20% of the expected mean count for the corresponding sequencing protocol	1[a]	Obtain another aliquot of the DNA library and subject to clustering and sequencing for a second attempt.
5	After sequencing	Number of sequenced reads showing correct index sequence	< 50% of the total sequenced read count obtained for the lane	10[b]	Obtain another aliquot of the DNA libraries and subject to clustering and sequencing for a second attempt.

QC, quality control; PCR, polymerase chain reaction

[a]During the 8-plex sequencing phase, one case that failed this criterion during the first run was sequenced again. During the second run, the case still failed the same criterion. The result was deemed invalid.

[b]During the 2-plex sequencing phase, 5 sequencing lanes (10 cases) that were distributed over four independent sequencing runs, failed this criterion. They were sequenced again on one slide in another run. All 10 samples passed the sequencing criterion and the results were deemed valid.

Appendix 2.
Supplementary Methods

Bioinformatics analysis

After sequencing was completed, a software package, named the ELAND (Efficient Large-Scale Alignment of Nucleotide Databases) programme (version 1.0 for the Genome Analyzer II and version 1.4 for the Genome Analyzer IIx), provided by Illumina, was used to identify the chromosomal origin of each sequenced DNA molecule. The software compares the DNA sequence of each molecule to the reference sequence of the human genome (Build 36 version 48, with the genomic regions containing repeats masked). The subsequent data analysis was performed with the use of an in-house bioinformatics algorithm developed by HS based on our previously reported strategy.[1] The algorithm automated all the steps for calculating the chromosome 21 z scores needed to determine if a sample would be classified as trisomy 21.

The proportion of reads from chromosome Y (%chrY) was also calculated and was used to determine the fetal DNA concentration in maternal plasma samples collected from pregnancies with male fetuses. We have previously reported that, due to a limitation of the current bioinformatics algorithms, a small number of DNA molecules would be incorrectly identified as originating from chromosome Y in pregnancies with female fetuses.[1] Hence, the maternal plasma %chrY value in a pregnancy with a male fetus is a composite of the amount of chromosome Y sequences contributed by the male fetus and those sequences from the maternal background DNA that were incorrectly assigned to chromosome Y. We determined that the mean %chrY value of plasma samples obtained from four adult male individuals (containing 100% male DNA) was 0.157%. The mean %chrY value in the plasma of all the women with euploid female fetuses (containing 100% female DNA) in this study was 0.007%. Thus, the fetal DNA concentration (F) can be derived from the equation: $chrY\% = 0.157F + 0.007(1-F)$.

Reference

1. Chiu RWK, Chan KCA, Gao Y, Lau VYM, Zheng W, Leung TY, et al. Noninvasive prenatal diagnosis of fetal chromosomal aneuploidy by massively parallel genomic sequencing of DNA in maternal plasma. *Proc Natl Acad Sci U S A* 2008;105:20458-63.

Appendix 3.

Profiles of the False Positive Cases.

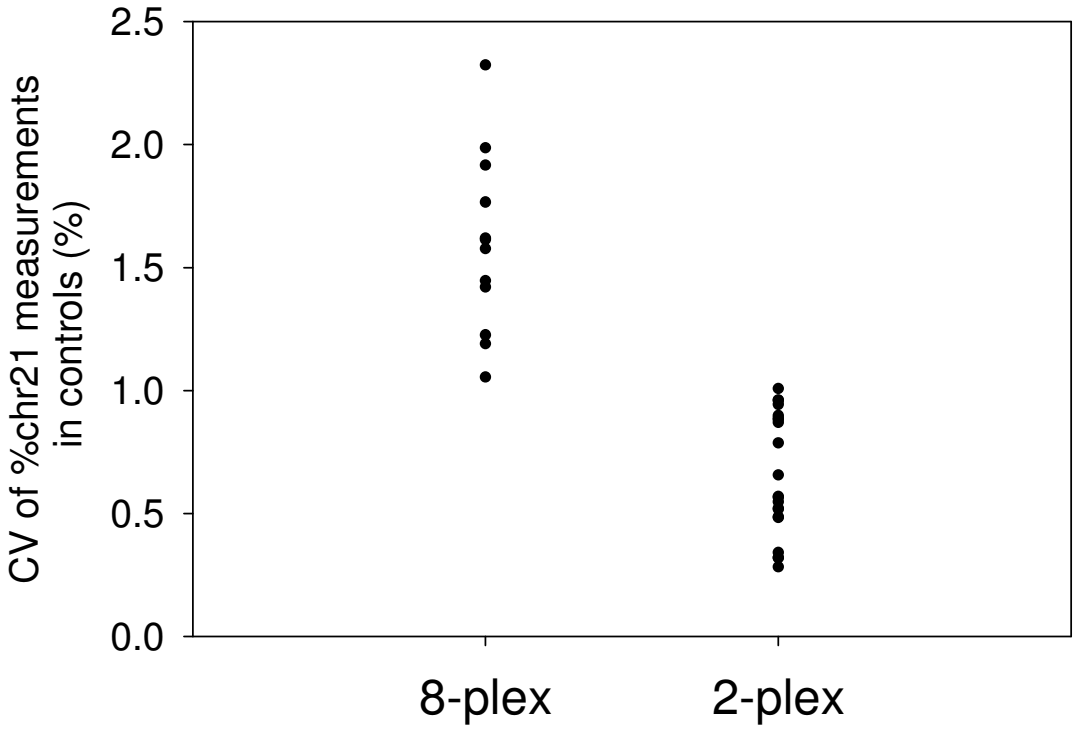
Sample No.	Source	Karyotype	Run No.[1]	MA (years)	GA	Risk[2]	chr21 z-score	Fetal DNA %
8-plex								
93619	UK	46XX	1	23	13 wk 1 day	1 in 261	3.06	NA
97032	UK	46XY	4	18	13 wk 4 days	1 in 30	4.27	14.7%
97259	UK	46XY	4	42	13 wk 4 days	Other	4.81	18.9%
97942	UK	46XX	5	34	14 wk 3 days	Other	3.59	NA
PW0025	HK	46XX	9	31	18 wk 1 day	1 in 37	3.43	NA
PW0078	HK	46XY	9	29	13 wk 4 days	1 in 280	4.40	10.3%
2-plex								
84863	UK	46XY	12	35	12 wk 3 days	Other	3.09	6.0%
2329	NL	46XX	22	38	13 wk 6 days	1 in 35	3.55	NA
2338	NL	46XY	22	35	9 wk 4 days	1 in 40	7.18	11.8%

MA, maternal age; GA, gestational age; UK, United Kingdom; HK, Hong Kong; NL, the Netherlands; wk, weeks; NA, not applicable.
[1]refers to the sequencing run number.
[2]refers to the clinical indication for invasive prenatal diagnosis as described in the main text.

Appendix 4.

Imprecision of sequencing measurements.

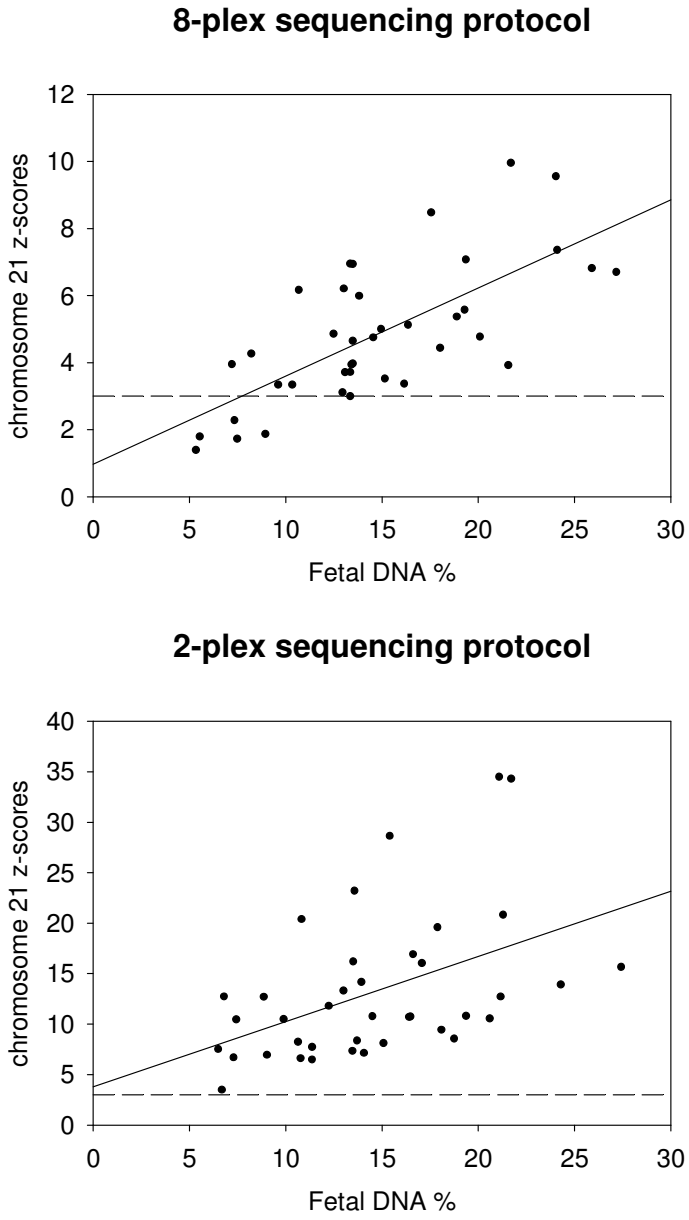
Coefficient of variation (CV) of the measurements of the proportion of DNA molecules from chromosome 21 (%chr21) among the controls on each sequencing glass slide analysed by the 8-plex or 2-plex sequencing protocols.



Appendix 5.

Correlation between chromosome 21 z scores and fetal DNA concentrations.

Dashed lines show the diagnostic cut-off at z score = 3.



Appendix 6.

Derivation of the equation to determine the analytical precision required to detect a particular degree of percentage chromosome 21 (%chr21) increment in trisomy 21 pregnancies at 99.9% confidence.

$$\text{mean } \%chr21_{\text{trisomy 21}} - \text{cut-off value} \geq 3SD,$$

where SD is the standard deviation of measuring %chr21 values among euploid pregnancies. As our pre-defined cut-off for differentiating trisomy 21 and euploid is at a z-score value of 3, the %chr21 cut-off value for identifying trisomy 21 is:

$$\text{mean } \%chr21_{\text{euploid}} + 3SD.$$

Therefore,

$$\text{mean } \%chr21_{\text{trisomy 21}} - (\text{mean } \%chr21_{\text{euploid}} + 3SD) \geq 3SD$$

Hence,

$$\text{mean } \%chr21_{\text{trisomy 21}} - \text{mean } \%chr21_{\text{euploid}} \geq 6SD$$

Thus, the threshold level in %chr21 increment for the diagnosis of trisomy 21 would be when:

$$\text{mean } \%chr21_{\text{trisomy 21}} - \text{mean } \%chr21_{\text{euploid}} = 6SD$$

As $SD = CV \times \text{mean } \%chr21_{\text{euploid}}$, the target CV, CV_t, required would be:

$$\text{mean } \%chr21_{\text{trisomy 21}} - \text{mean } \%chr21_{\text{euploid}} = 6 \times CV_t \times \text{mean } \%chr21_{\text{euploid}}$$

Let Δ be $\text{mean } \%chr21_{\text{trisomy 21}} - \text{mean } \%chr21_{\text{euploid}}$. When $\text{mean } \%chr21_{\text{euploid}} = 1.264\%$, the expected %chr21 for a maternal plasma sample obtained from a trisomy 21 pregnancy containing 10% fetal DNA is $1.264\% \times 1.05 = 1.327\%$. Therefore,

$$\Delta = 1.327\% - 1.264\% = 6 \times CV_t \times 1.264\%$$

$$0.063\% = 6 \times CV_t \times 1.264\%$$

$$CV_t = 0.83\%$$

Appendix 7.

Proportion of chromosome Y sequences in pregnancies involving male fetuses, female fetuses and fetuses with sex chromosome mosaicism.

Dashed lines indicate the cut-off values identified by receiver-operating characteristic curve analysis for detecting male and female fetuses.